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Toxin tolerance across landscapes: Ecological exposure not a prerequisite

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Abstract

1. Little is known about the tolerances of mammalian herbivores to plant specialized metabolites across landscapes.

2. We investigated the tolerances of two species of herbivorous woodrats, *Neotoma lepida* (desert woodrat) and *Neotoma bryanti* (Bryant's woodrat) to creosote bush (*Larrea tridentata*), a widely distributed shrub with a highly toxic resin. Woodrats were sampled from 13 locations both with and without creosote bush across a 900 km transect in the US southwest. We tested whether these woodrat populations consume creosote bush using plant metabarcoding of feces and quantified their tolerance to creosote bush through feeding trials using chow amended with creosote resin.

3. Toxin tolerance was analyzed in the context of population structure across collection sites with microsatellite analyses. Genetic differentiation among woodrats collected from different locations was minimal within either species. Tolerance differed substantially between the two species, with *N. lepida* persisting 20% longer than *N. bryanti* in feeding trials with creosote resin. Furthermore, in both species, tolerance to creosote resin was similar among woodrats near or within creosote bush habitat. In both species, woodrats collected greater than 25 km from creosote had markedly lower

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tolerances to creosote resin compared to animals from within the range of creosote bush.

4. The results imply that mammalian herbivores are adapted to the specialized metabolites of plants in their diet, and that this tolerance can extend several kilometers outside of the range of dietary items. That is, direct ecological exposure to the specialized chemistry of particular plant species is not a prerequisite for tolerance to these compounds. These findings lay the groundwork for additional studies to investigate the genetic mechanisms underlying toxin tolerance and to identify how these mechanisms are maintained across landscape-level scales in mammalian herbivores.

Keywords

creosote bush; herbivory; Mojave Desert; toxin tolerance; woodrats

Introduction:

The interactions between herbivores and the plants on which they feed often drive the evolution of traits present in one or both parties (Crawley, 1983). To defend themselves from herbivory, plants rely on a variety of defenses including the production of specialized metabolites (SMs), many of which can be highly toxic. In response, herbivores have evolved mechanisms to tolerate and metabolize plant chemistry, such as biotransformation enzymes, efflux transporters, and specialized gut microbiomes (Forbey & Foley, 2009; Freeland & Janzen, 1974; Futuyma, 2000; Kohl et al., 2014). Ultimately, the co-evolutionary arms race between increasingly toxic plants (or novel toxins) and toxin-tolerant herbivores can result in the local adaption of herbivores to the specialized chemistry of the plants that constitute their diet (Fox & Morrow, 1981; O'Connor et al., 2019).

Invertebrate herbivores often exhibit local adaptation to plant chemistry across landscape-level scales, and invertebrate tolerance of SMs has been widely studied across broad geographical regions (Bradley et al., 2018; Fox et al., 1994; Fox & Morrow, 1981; Jaenike, 1990; Kuussaari et al., 2000; O'Connor et al., 2019). In contrast, an understanding of how mammalian herbivores tolerate SMs is restricted to a few species within limited geographical ranges (DeGabriel et al., 2009; Mangione et al., 2001). The lack of landscape scale surveys in mammals is driven by many factors, including restricted range distributions of plant and herbivore species, challenges in obtaining sufficient sample sizes, and the limited information on mammalian herbivore diets and plant chemistry. These limitations are further compounded by the difficulties and costs of working with mammals under laboratory conditions. Thus, while tolerance to SMs in mammalian herbivores may be shaped by the local chemical landscape, this hypothesis remains largely untested. It is critical to understand the abilities of mammalian herbivores to adapt to their local environment on a broader scale as the accelerating impacts of climate change will likely alter plant composition and chemistry across continents.

Woodrats (*Neotoma*) provide a unique opportunity to examine SM tolerance at a landscape-level scale in mammalian herbivores. With large species distributions, prior work on dietary habits, and suitability for captivity, they represent a tractable system for such a study. We focused on the desert woodrat (*Neotoma lepida* Thomas) and the Bryant's woodrat (*N. bryanti* Merriam) for this work. These species diverged approximately 1.5 mya (Patton et al., 2008), and independently incorporated creosote bush (*Larrea tridentata*) into their diets sometime after the end of the last glacial maximum (approximately 18 kya) as this plant expanded across the Sonoran and Mojave Deserts (Betancourt et al., 1990; Hunter et al., 2001; Spaulding, 1990). Few herbivores feed on creosote bush due to its toxicity (Rundel et al., 1994), despite it often being the dominant species across millions of hectares of North American desert (Barbour et al., 1977; Wells & Hunziker, 1976). Creosote bush is renowned for its exceptional investment in defensive chemistry, with 15% or more of its leaf dry mass allocated to a chemically complex resin (Rhoades & Cates, 1976). This resin contains more than 300 compounds (Mabry et al., 1977), many of which are toxic to mammals (Arteaga et al., 2005). The most abundant compound is nordihydroguaiaretic acid (NDGA) — a particularly toxic phenolic compound that causes kidney and liver disease (Arteaga et al., 2005). Both *N. bryanti* and *N. lepida* naturally consume the plant when within creosote bush habitat, but there is far more overlap with the large range of *N. lepida* in the US (Figure 1A). Seasonally, creosote bush may be one of only a few live plants in these habitats, and it is unknown whether the ability to tolerate creosote bush toxicity has allowed *N. lepida* to succeed in this range.

Notably, woodrats collected from different sites differ in their ability to tolerate creosote resin. The ability of *N. lepida* to consume substantial amounts of creosote bush was first demonstrated in animals collected near Barstow, CA, where creosote bush is abundant (Karasov, 1989; Meyer & Karasov, 1989). Follow-up studies on *N. lepida* collected from a site with creosote bush and a second site several hundred kilometers outside of its range (Kohl et al., 2014; Mangione et al., 2000, 2001, 2004) showed that woodrats from within the range of the plant could ingest more creosote resin and persist on the plant for longer than conspecifics with no prior exposure. Less is known about *N. bryanti*'s ability to ingest creosote bush; however, their tolerance to low levels of creosote resin may be comparable to *N. lepida* (Malenke et al., 2014). For both species, the ability to feed on creosote resin is in part mediated by several of the major groups of biotransformation enzymes expressed in the liver (Haley et al., 2008; Lamb et al., 2001; Malenke et al., 2014; Mangione et al., 2000, 2001).

To advance a general understanding of local adaptation in mammalian herbivores, we studied tolerance to creosote bush in *N. bryanti* and *N. lepida* collected from 13 locations ranging from coastal southern California to northern Utah (a distance of approximately 900 km). We collected individuals of both species in habitats with and without creosote bush to 1) test whether both species tolerate creosote bush equally, and 2) determine the extent to which ecological exposure explains variation in toxin tolerance both within and between woodrat species. To study the potential for local adaption to toxic diets, we confirmed creosote bush feeding in nature using diet metabarcoding, interrogated population structure with microsatellites, and experimentally quantified tolerance to creosote resin with diet trials.

Materials and methods:

Animal capture and maintenance

We collected woodrats at 13 sites in California, Nevada and Utah, selecting sites based on collection records of these two species (Figure 1A, Table 1; Mangione et al., 2000, 2001, 2004; Patton et al., 2008). We categorize sites as either within, near or far from creosote bush; creosote bush range is limited by climate and elevation (less than 1,500 m; Marshall, 1995). We collected *N. lepida* in habitats with creosote bush at six locations; sites with creosote bush present are indicated with an asterisk. The overlap between *N. bryanti* and creosote bush is more limited, and human development has caused local extirpation of some populations. As a result, we only collected *N. bryanti* from one location with creosote bush (CA-WW*). Additionally, to limit the effects of population structure, we constrained our sampling of *N. bryanti* and *N. lepida* to only sampling localities within the same mtDNA *cyt-b* subclades previously identified by Patton et al. (2008). At each site, we collected 7–18 individuals of each species present using long Sherman live traps (7.6 × 8.9 × 22.9 cm, HB Sherman Traps Inc.) baited with oats and peanut butter. At capture, we sexed and weighed animals, and collected feces and an ear clip tissue sample. We transported live woodrats to the animal facility in the School of Biological Sciences at the University of Utah, where animals were maintained on a 12L/12D light cycle at 24°C and 15–20% relative humidity. At the facility, individuals were housed singly in solid-bottom shoebox cages (48 × 27 × 20 cm) with pine shavings and a plastic tube for shelter. Animals received pelleted high-fiber rabbit chow (Harlan Teklad formula 2031) and water *ad libitum* while acclimating to captivity for approximately three weeks prior to the creosote resin diet trials. All animal work was approved under the University of Utah's Institutional Animal Care and Use Committee (Protocol 16–02011) and by CA Department of Fish and Wildlife Scientific Collection Permit (SC-8123), Nevada Department of Wildlife scientific collection permit (LN-333663), and Utah Division of Wildlife Resources Collect/Possess/Release permit (5194–1). Research at Death Valley National Park, CA was conducted in collaboration with Dr. James Patton (SC-2105).

Characterizing natural diets

To characterize woodrat diets at each site and confirm the extent to which animals within creosote bush habitat ingest creosote bush, we sequenced the plant material in feces collected at the time of capture. Fecal samples were stored on dry ice in the field and held at −80°C prior to extracting DNA. We isolated DNA using QIAamp PowerFecal DNA kits (Qiagen: 12830) following manufacturer protocols. To estimate dietary components, part of the chloroplast *trn*L (UAA) intron was amplified using the *g* and *h* primers (Taberlet et al., 2007). Extracted DNA was processed for sequencing at the DNA Service Facility at the University of Illinois-Chicago and libraries were sequenced using the Illumina MiniSeq platform (150 bp paired-end reads). Illumina sequencing reads were processed with DADA2 v.1.14.1, using Cutadapt v.2.9 to remove primers and phyloseq v.1.30.0 for additional filtering (Callahan et al., 2016; Martin, 2011; McMurdie & Holmes, 2013). To assign plant taxonomy to *trn*L amplicons, we constructed a reference database using sequence and taxonomy data downloaded from NCBI. To accommodate *trn*L amplicon lengths (Taberlet et al., 2007), which are often shorter than the minimum sequence length required in programs

like DADA2, and to account for plant geographic distributions, we assigned taxonomy using a custom Python script (https://github.com/robertgreenhalgh/stand), as described in Weinstein et al. (2021). We removed amplicon sequence variants likely derived from bait (*Avena* (oats), *Glycine* (soy), or *Arachis* (peanut)), and for each animal, removed any amplicon sequence variants representing less than 1% of the diet (Ando et al., 2018).

Species assignment and population structure

In areas of sympatry, *N. bryanti* and *N. lepida* are morphologically indistinguishable (Patton et al., 2008); therefore, we used microsatellites to identify woodrats to species. We extracted DNA from ear-clip tissue with the DNeasy Blood & Tissue Kit (Qiagen: 69504), and genotyped animals at 17 microsatellite loci developed specifically for *Neotoma* spp. (Sousa et al., 2007). Amplification procedures followed Coyner et al. (2015), and Nielsen and Matocq (2021); products were run on an Applied Biosystems Prism 3730 DNA Analyzer, and peaks were called using GeneMarker v.3.7. We established species identity for an initial subset of 25 individuals from this study by combining their genotypes with those of a set of known *N. bryanti* (N = 40) and *N. lepida* (N = 40) individuals from Mojave Desert populations reported in Nielsen et al. (2021). Genotypes were analyzed with STRUCTURE v.2.3.4 (Falush et al., 2003; Pritchard et al., 2000) using the admixture model of ancestry and the independent allele frequency model. We ran 10 iterations of K = 2 using a burn-in of 1×10^4 steps followed by 1×10^5 Markov Chain Monte Carlo replicates as in Shurtliff et al. (2014) and Nielsen and Matocq (2021). We identified individuals with $q_{lepida} > 0.90$ as *N. lepida*, $q_{lepida} < 0.10$ as *N. bryanti*, and intermediate q values as hybrids. Subsequent analyses of the entire dataset presented herein were run as above. We did not analyze microsatellites from animals from NV-IS* and NV-KC. Both of these sites are over 200 km outside of the *N. bryanti* range, and thus the species identity of these samples is known as *N. lepida* without microsatellite analysis.

Pairwise-F_{ST} calculations and modeling genetic isolation by distance

The microsatellite data were imported into R v.4.0.1 (R Core Team, 2021) and converted into an allele frequency matrix with the R package adegenet v.2.1.3 (Jombart, 2008). Using the R package HIERFSTAT v.0.5–7 (Goudet, 2005), pairwise fixation-indices (F_{ST}) were calculated with associated 95% confidence intervals derived from 1,000 bootstraps using the Weir and Cockerham equation (Weir & Cockerham, 1984) between all sampling sites of both woodrat species. A linear regression model was calculated using stats v.4.0.1 to predict the relationship between geographic distance (pairwise site distance in kilometers) and genetic isolation (pairwise fixation indices, F_{ST}) across woodrat sampling sites for both species.

Diet preparation for feeding trials

To prepare diets for feeding trials, we collected creosote foliage from six sites at the same time we collected woodrats (Table 1). To preserve the SMs in creosote bush, we placed plant material collected from a minimum of 15 separate creosote plants in sealed plastic bags, transported it on dry ice, and then stored it at −20°C until resin extraction. Resin was extracted from creosote leaves following established methods (Kohl et al., 2014). Foliage was removed from branches and leaves were soaked in acetone (1:6 wet leaf mass: volume

solvent) for one hour. The extract was filtered using a Buchner funnel (Coors® #60247) and 185 mm Whatman filter paper (1001-185) under a low vacuum. Acetone was evaporated from the extract using a rotary evaporator until the resin was highly viscous, which required about 14 hours. To calculate the percentage of the creosote bush dry mass allocated to resin, we divided the final resin mass by the initial plant dry matter. The majority of extractions resulted in yields of approximately 15% resin by dry weight, which is the amount typically found on the mature leaves of creosote bush (Rhoades & Cates, 1976). Resin was stored at −20°C until diet preparation.

We used this extracted resin to prepare experimental diets by dissolving resin into acetone at a volume of acetone equal to 25% of the total diet being prepared. The resin-acetone solution was mixed with ground rabbit chow (Harlan Teklad formula 2031) until homogeneous. Acetone was evaporated in a fume hood for 24 hours and then placed under vacuum for at least one hour to remove any remaining solvent prior to use in trials. We prepared diets in a powdered form to prevent woodrats from caching food. Diets were stored in sealed plastic bags at −20°C until use.

Feeding trials

To determine tolerance, we conducted a challenge trial where we offered woodrats diets with increasing levels of creosote resin (Kohl et al., 2014; Mangione et al., 2000, 2004). As diets increase in toxicity, herbivores will often prioritize the regulation of food intake over the preservation of body mass, such that they consume less food and subsequently lose mass as toxin concentration increases (Foley & McArthur, 1994; Torregrossa et al., 2012). As a result of this behavior, gradually increasing the toxin dosage while monitoring animal body mass provides an accurate and non-lethal method for assessing differences in toxin tolerance. As losing more than 10% of their body weight is often lethal in woodrats (Karasov, 1989), animals were weighed daily and each individual's trial ended when the animal lost more than 9.5% of its starting mass.

All diet trials began with two days of ground rabbit chow, after which woodrats were presented daily with known amounts of creosote resin diets in excess of maintenance levels in stainless steel feeder hoods (Lab Products, LLC.). We gradually increased the amount of resin in the diet (1%, 2%, 3%, 4%, 6%, 9%, 12%, 15%) and fed each concentration for two days to permit adequate time for the induction of hepatic biotransformation enzymes (Klaassen, 2001), and to prevent radical reductions in food intake and loss of body mass. This is a standard protocol used in our laboratory and others (e.g., Marsh et al., 2005; Skopec et al., 2015). Animals from sites with creosote bush were given diets with resin extracted from the creosote bush at their site. For trials with *N. lepida* from sites without creosote bush, we used resin extracted from nearby sites (Table 1). Since site CA-PT was situated close to both CA-WW* and CA-HP*, 4 animals from CA-PT were given resin extracted from plants taken from CA-WW* whereas the remaining 3 animals were given resin from CA-HP*. For *N. bryanti* from sites without creosote bush, we used CA-WW* creosote resin to compare all *N. bryanti* on the same resin. We measured daily food intake for all animals, however, some individuals displayed feeding behaviors that biased their food

intake data. Therefore, to retain data from all animals we used persistence in the diet trial to measure tolerance.

We analyzed the data from the tolerance trial using Kaplan-Meier survivorship analysis (Wilcoxon test) with right-censoring for animals that persisted for the entire trial, i.e., never lost more than 9.5% body mass during the study. Persistence (i.e., "survival") in the trials was compared between the creosote-feeding *N. bryanti* (N = 18) and *N. lepida* (N = 64), and also between *N. lepida* (N = 45) and *N. bryanti* (N = 40) from sites without creosote bush. Additionally, persistence was compared among the *N. lepida* with creosote bush present at the collection site to determine if there were differences in tolerance among woodrats that could naturally feed on creosote bush. We conducted survivorship analyses in JMP Pro v.15.2.0, corrected for multiple comparisons using Holm's method with the p.adjust function in R v.3.6.3, and report averages as mean (± 1 standard error; SE).

Results:

Species identity

Using an initial subset of 25 individuals in comparison to known *N. lepida* and *N. bryanti* collected north of our study area in the Mojave Desert (Nielsen & Matocq, 2021), we identified 8 *N. bryanti* and 17 *N. lepida*. Using these individuals as "known" reference samples, we assigned our study animals as *N. bryanti* or *N. lepida*. Our species assignments agreed with expectations from previous collections and genetic assignments made at these and nearby sites (Patton et al., 2008). Using these methods, several (N = 15) hybrids were also identified. Due to low hybrid numbers from only a few sites (CA-WW*, CA-PT, CA-DC*), and to eliminate variance caused by the hybrid phenotype, these animals were not included in this study.

Natural diets

Woodrats consumed a variety of plants, with creosote bush found in the diets of nearly all woodrats sampled at sites with creosote bush present (Figures 1B, S1). The average relative read abundance of creosote bush DNA in woodrat feces varied from 6−52% across sites (Figure 1B). No evidence of creosote bush ingestion was found in woodrats at sites lacking creosote bush, even at sites within only a few kilometers of the nearest creosote bush habitat (e.g., CA-PT; CA-FC).

Population structure within species

Pairwise F_{ST} values suggest that there is minimal genetic differentiation across most sampling sites within each species (Tables S1, S2). For *N. lepida*, pairwise F_{ST} values ranged from 0.008 to 0.099, with the highest F_{ST} values found between animals separated by the largest geographic distances. In our most extreme geographic comparison, *N. lepida* from the southernmost site in California (CA-OW*) were only modestly differentiated from the conspecifics at the northernmost site in Utah (UT-WR) (F_{ST} = 0.099). For *N. bryanti*, pairwise F_{ST} values ranged from 0.032 to 0.065. Pairwise fixation indices and pairwise site geographic distance in kilometers were positively correlated for *N. lepida* ($F_{1:34}$ = 5.8, P = 0.002, R^2 = 0.15), but not for *N. bryanti* (P = 0.31). The F_{ST} values increased by

0.0036 per 100 km of distance between *N. lepida* sampling sites (Figure S2). These results are consistent with genetic isolation by distance and are not indicative of the presence of discrete populations of *N. lepida*, at least for those in this study.

Tolerance to creosote resin

Neotoma bryanti and *N. lepida* from creosote bush habitat differed from each other in their ability to tolerate creosote resin (Wilcoxon: $\chi^2 = 29.2$, $P < 0.0001$, Figure 2A). In these groups, *N. bryanti* persisted for ~20% less time (12.6 days ± 0.6) in laboratory feeding trials than *N. lepida* (15.1 days ± 0.2). Creosote-feeding *N. bryanti* began dropping out of the trial as early as day 9 on 4% resin, compared to creosote-feeding *N. lepida* that only began dropping out at day 12 on 9% resin.

Neotoma bryanti sampled from sites within and near creosote bush habitat had similar tolerances, while those from far outside creosote bush's range did not (Figure 2B). Animals from the sites near creosote bush habitat, CA-PT and CA-RL, persisted on average for the same number of days as the creosote-feeding *N. bryanti* from CA-WW* ($P > 0.51$ for all pairwise comparisons; Table S3). In contrast, animals from CA-CW, the location farthest outside creosote bush's range, persisted for a significantly shorter time (10 days ± 0.6) than animals from the other three sites (combined 12.6 days ± 0.5; $P < 0.008$ for all pairwise comparisons; Table S4). All *N. bryanti* regardless of distance from creosote bush habitat dropped out before the end of the 16-day trial.

Similar to *N. bryanti*, *N. lepida* from sites within and near creosote bush habitat had similar tolerances to creosote resin (Figure 2C–F). No *N. lepida* sampled from sites over 25 km from the nearest creosote bush habitat persisted throughout the diet trial (UT-EN, UT-WR; Figure 2C), while 20% of those sampled less than 25 km from creosote bush habitat (Figure 2D, E), and 38% of those within creosote bush habitat (Figure 2F), persisted throughout the trial. When comparing *N. lepida* fed creosote from the same source, we found that animals sampled from far outside the range of creosote bush had lower tolerances than animals from the creosote sampling site. These animals were all fed UT-LR* creosote, and UT-LR* persisted ~30% longer (15 days ± 0.4) than UT-EN and UT-WR (combined 10.1 days ± 0.5; Wilcoxon: $\chi^2 = 18.6$, $P < 0.0001$; Figure 2C). UT-EN and UT-WR did not differ from each other (Wilcoxon: $Z = 0.19$, $P = 0.66$). In contrast, there was no significant difference in persistence among woodrats from the three sites fed creosote resin from NV-IS* (Figure 2D; Wilcoxon: $\chi^2 = 6.0$, $P = 0.05$; no difference across pairwise comparisons), nor those fed creosote resin from either CA-HP* or CA-WW* (sites located adjacent to one another) — this includes 7 animals from CA-PT that were split between the two creosote sources (Figure 2E; Wilcoxon: $\chi^2 = 6.0$, $P = 0.09$).

There were, however, differences in persistence among the creosote-feeding *N. lepida* collected from different locations (Wilcoxon: $\chi^2 = 20.8$, $P < 0.0009$; Figure 2F). Of all sites with creosote, CA-HP* had the most animals completing the trial at 85.7%, while the least tolerant group was NV-IS*, where all animals dropped out. Woodrats from CA-HP* had significantly higher persistence than NV-IS*, CA-DC* and UT-LR*, but no other pairwise comparisons among the creosote-feeding *N. lepida* were significant (Table S4). For *N. lepida* that persisted throughout the diet trial, animals lost an average of only 3.7% (± 0.6) of their

starting mass even when feeding on a diet of 15% creosote resin. There was no difference in the average starting mass of *N. lepida* that persisted throughout the trial compared to those that did not ($F_{1,63}$ = 0.83, P = 0.36). Starting mass varied between species, with *N. bryanti* (132.8 ± 6.0 g) being approximately 18% larger than *N. lepida* (111.7 ± 3.2 g; $F_{1,81}$= 9.1, P = 0.004).

Discussion

This work sought to expand our understanding of local adaptation in plant-mammal interactions by quantifying the landscape scale variation in toxin tolerance of two mammalian herbivore species. We found three notable outcomes. First, *N. lepida* and *N. bryanti* that naturally consume creosote bush differ in their tolerance to creosote resin. Second, in both species, current ecological exposure to creosote bush (i.e., living in creosote bush habitat) did not predict tolerance, as measured by persistence. Third, the resin tolerances across the range of sampled *N. lepida* were remarkably similar within or near creosote bush habitat. Below we discuss each of these findings, how they may have been shaped by each species' evolutionary experience with creosote bush, and their relevance with respect to the future of these species under impending habitat alterations and projected climate change.

Neotoma lepida *has a higher tolerance to creosote toxins than* N. bryanti

Despite both *N. bryanti* and *N. lepida* having geographical overlap with the range of creosote bush, the latter could consume significantly more creosote resin and for longer periods of time than the former. Moreover, nearly 40% of creosote-feeding *N. lepida* persisted to the end of diet trial, losing little body mass while feeding on a final concentration of resin approximately equal to a diet of 100% mature creosote leaves (10–15% resin; Mabry et al., 1977). In contrast, none of the *N. bryanti* persisted to the end of the trial. *Neotoma bryanti*'s lower tolerance in laboratory feeding trials implies a lesser ability to rely on creosote bush foliage at times when other plants are not available, and thus might result in a larger nutritional bottleneck for this species during periods of food scarcity than that experienced by *N. lepida* (Karasov, 1989).

The lower tolerance of creosote-feeding *N. bryanti* to creosote resin was unexpected for several reasons. Our only site (CA-WW*) where both creosote bush and *N. bryanti* were present was one where creosote bush is by far the most abundant food item in terms of available plant biomass, and is likely an important food resource year-round (Figures S1, S3). *Neotoma lepida*, with its greater tolerance to creosote bush, was also present at this site; however, *N. bryanti* was much more common than *N. lepida*, and was captured more than 2.5 times as often (Table 1). We do not know of any source of bias in the capture of these two species that would lead to this outcome and therefore interpret the difference in the number of woodrats trapped per species to be representative of their frequencies at this site. Our dietary analysis suggests that both species of woodrats at CA-WW* ingested similar amounts of creosote bush at the time of collection, which constituted approximately 40–50% of the relative read abundance for both species. However, given the inaccuracies associated with using relative read abundances to precisely quantify individual dietary components

(Deagle et al., 2019; Stapleton et al., *In Press*), more work is needed to confirm this pattern. Additionally, consistent with patterns observed at other sympatric sites (Nielsen & Matocq, 2021), CA-WW* *N. bryanti* appeared to consume a more diverse diet than *N. lepida*, although the smaller sample sizes for the latter might drive this apparent difference in dietary diversity (Figure S1). Nonetheless, our dietary analysis coupled with poorer persistence in the feeding trials suggest that *N. bryanti* may cope with creosote-dominated habitat by dietary diversification while *N. lepida* could persist on greater proportions of creosote. It is also possible that *N. bryanti* may selectively forage on creosote bush that is less toxic. We are currently investigating how *N. bryanti* maintains its numerical dominance in this creosote-dominated habitat where *N. lepida* would seem to have an advantage during dry months, when the plant community becomes restricted to few plants, including creosote bush.

Current ecological exposure to creosote bush is not required for tolerance

Within each species, residing in a habitat with creosote bush was notably not a prerequisite for higher tolerance to creosote resin. In both species, woodrats that lacked direct exposure to creosote bush, but were caught less than 25 km from creosote bush habitat, had resin tolerances comparable to woodrats that feed upon creosote bush. Creosote bush growth is elevation-limited (less than 1,500 m) enabling distinct delineation of woodrat populations at higher elevations as having no ecological exposure, even along the borders of its range (Marshall, 1995). Woodrats are capable of traveling several kilometers in a single night and even further when dispersing (Djawdan & Garland, 1988; Sorensen et al., 2005). Therefore, it is possible that at sites near creosote bush habitat, we trapped creosote-feeding animals or animals that were the product of admixture with creosote-feeding populations. In fact, the distinct lack of population structure as evidenced by our microsatellite analysis strongly suggests that these populations are largely panmictic. Thus, it is also possible that the higher tolerance of woodrats with no prior creosote experience is the result of interbreeding with neighboring creosote-feeding conspecifics.

We acknowledge that the evidence for local adaption in *N. bryanti* is more limited than that observed in *N. lepida*. Although we sampled *N. bryanti* at four localities, only one occurred more than 25 km from the range of creosote bush and exhibited a lower tolerance to its resin. Thus, additional sampling of animals far outside the range of creosote bush is needed to establish whether this result is limited to the particular populations sampled or is a general pattern among *N. bryanti*.

Toxin tolerance is consistent in *N. lepida* within the range of creosote bush

Neotoma lepida that naturally feed on creosote bush exhibited remarkably consistent tolerances across seven localities spanning 650 km (Figure 2F). The majority of sites had individuals with similar resin tolerances that persisted throughout the 16-day diet trial, with concentrations roughly equivalent to earlier findings suggesting desert woodrats can survive on a diet of 75% creosote bush in nature for extended periods of time (Karasov, 1989; Meyer & Karasov, 1989). *Neotoma lepida* from CA-HP* had notably higher tolerances. This result was unexpected given that this site has a high diversity of plant species, some with biomass available year-round (e.g., *Yucca, Opuntia*). The plant diversity at this site was

reflected in the aggregated diet as being the broadest among the creosote feeders and with very low levels of creosote bush in the diet (Figures 1B, S1). This site was adjacent to other creosote-dominated sites such as CA-WW*, so it is conceivable that interbreeding among *N. lepida* across sites — as indicated by the lack of distinct population structure — provides the appropriate mechanisms to enable high levels of creosote tolerance. Additionally, it is possible that the higher tolerance of CA-HP* woodrats was the result of a reduced resin toxicity, since creosote resin components vary across landscapes (Rundel et al., 1994). Understanding whether the differential tolerance of the woodrats at CA-HP* is a result of higher tolerance capacities of the woodrats at that site, lower toxicity of the creosote bushes at that site (or time), some combination of both, or an entirely different mechanism (e.g., differences in the capacity of the gut microbiome; Kohl et al., 2014), presents opportunities for future work.

Temporal differences in creosote bush exposure may partially explain these results

Compared to *N. bryanti*, longer evolutionary exposure to creosote bush may be a key contributor to *N. lepida*'s higher tolerance to creosote bush toxins. The current distribution of *N. lepida* overlaps with creosote bush to a far greater extent than that of *N. bryanti* (Figure 1A) (Patton et al., 2008), and many of the locations where *N. lepida* occurs are dominated by creosote bush (e.g., CA-OW*, CA-WW*, others listed in Patton et al., 2008), perhaps exacting greater selection pressure than in other desert habitats with higher plant diversity. In contrast, *N. bryanti*'s overlap with creosote bush is more geographically limited and confined to sites located in or near Morongo Valley, CA (Patton et al., 2008).

Despite *N. lepida*'s high tolerance to creosote resin, the majority of the individuals in this study could not persist without substantial mass loss on resin levels comparable to a diet of exclusively creosote leaves. While selective feeding on leaves with lower resin content along with supplemental food from caches might facilitate survival under these conditions, *N. lepida* still experiences a nutritional bottleneck during winter and experiences significant mortality when creosote bush is the only widely available food item with sufficient water content (Karasov, 1989). This nutritional constraint likely selects for individuals with a high tolerance to creosote resin (Karasov, 1989).

Lastly, *N. lepida* seems to have been exposed to creosote bush for a longer period of time, based on its proposed historical distribution. The point of origin of the initial colonization of creosote bush in North America remains unknown; however, current palaeobotanical evidence suggests that creosote bush was first in southern Mexico sometime in the transition period of the Pleistocene to the Holocene, and expanded northward from Mexico across the southwestern US approximately 10–15 kya (Wells & Hunziker, 1976). These distribution estimates are, in part, derived from creosote bush samples present in the paleocaches of *Neotoma* spp. (Hunter et al., 2001; Wells & Hunziker, 1976), showing historic interaction within the ancestral range of *N. lepida*. In contrast, there is less evidence for historical interactions between *N. bryanti* and creosote bush outside of narrow portions of the southern Mojave, as *N. bryanti* predominately occurs within coastal and inland regions of California and Baja Sur where creosote bush habitat is largely absent. While an exact estimate of the temporal difference in exposure to creosote bush between the two species is not currently

possible, the difference could be on the order of several millennia, giving *N. lepida* more time to develop endogenous mechanisms for creosote toxin tolerance.

The adaptations responsible for creosote toxin tolerance are not yet known

Although the specific mechanisms underlying *N. lepida*'s superior performance in the creosote tolerance trials are not fully understood, previous work yields potential explanations for this outcome. Gut microbes can enhance *N. lepida*'s tolerance of creosote (Kohl & Dearing, 2012); however, this mechanism is unlikely to have played a substantial role in this study. Animals consumed a non-toxic chow prior to the resin challenge and this diet treatment results in a reduction or apparent loss of the microbes correlated with particular plant diets, including creosote bush (Martínez-Mota et al., 2020; Weinstein et al., 2021). It is therefore more likely that the tolerance to creosote resin was the result of several hepatic detoxification enzymes (e.g., cytochrome P450 monooxygenases — hereafter P450s, and UDP-glucuronosyltransferases that have been identified in previous studies (Haley et al., 2008; Lamb et al., 2001; Malenke et al., 2014; Mangione et al., 2000, 2001)). P450s in particular have been implicated in the evolution of tolerance to the toxic SMs of several plant species, including in invertebrates such as the Solanaceae-feeding Colorado potato beetle (*Leptinotarsa decemlineata*; Zhu et al., 2016), and vertebrates such as the eucalyptus-feeding koala (*Phascolarctos cinereus*; Iason & Villalba, 2006) and juniper-feeding *N. lepida* (Malenke et al., 2012). We are currently engaged in additional genomic and transcriptomic studies to identify the particular mechanisms enabling high tolerance to creosote resin in woodrats.

Climate change and habitat alteration will likely accelerate existing trends

Ongoing anthropogenic climate change is predicted to increase temperatures, aridity, and desertification in the US southwest, leading to an increase in the distribution of creosote bush across the landscape (Brown & Minnich, 1986; Munson et al., 2012, 2014). Such anticipated floristic changes are already being documented across the range of creosote bush (Thomey et al., 2014). Woodrats in general, and *N. lepida* in particular, may be able to capitalize on this event due to their high tolerance for creosote bush, their ability to buffer themselves from ambient temperature fluctuations through midden construction (Riddell et al., 2021), and their history of adaptation to natural climate change (Smith et al., 2014; Tomé et al., 2021). However, *N. lepida* exhibit lower tolerances to creosote resin at higher ambient temperatures, possibly due to the tradeoff between thermal regulation and detoxification (Connors et al., 2017; Kurnath et al., 2016). Additionally, creosote bush may also become more toxic under elevated CO_2, since its resin is comprised of many carbon-based compounds that could potentially increase with higher levels of atmospheric carbon (Stiling & Cornelissen, 2007). Thus, it is difficult to predict how creosote-feeding woodrats will respond to impending changes in climate.

It is also worth noting that the current distribution of *N. bryanti* in creosote-dominated landscapes may be contracting compared to previous records. Despite extensive trapping efforts, we were unable to obtain *N. bryanti* at two creosote bush sites (CA-DC* and CA-HP*) where they had previously occurred (Patton et al., 2008). Additionally, we identified *N. lepida* occurring in sympatry with *N. bryanti* at CA-WW* (a site on the far western edge

of creosote bush's range in the Mojave Desert) where *N. lepida* was not previously found (Patton et al., 2008). Climate change has been implicated in both facilitating range shifts and the formation of new sympatric zones between other woodrat species (Hunter et al., 2017; Moritz et al., 2008); as such, it is possible that *N. lepida* may be expanding westward into the range of *N. bryanti* in southern California as these regions experience aridification (Cook et al., 2015). This, coupled with the fact that the range of *N. bryanti* is more fragmented than *N. lepida* due to its overlap with heavily urbanized regions in California — as well as its lesser ability to tolerate creosote bush toxins — could indicate that *N. bryanti* may be at greater risk for population and range declines secondary to climate change. A more thorough investigation of the distribution of *N. bryanti* is therefore warranted to assess this species' current and future population trends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability:

Data used for the survival analyses is available from https://doi.org/10.17605/osf.io/3crwb. The sequences data used for wild diet analyses were obtained from Weinstein, et al. 2021. *PNAS*. 118 (47) e2108787118, and are available from the NCBI SRA under Bioproject: PRJNA722312.

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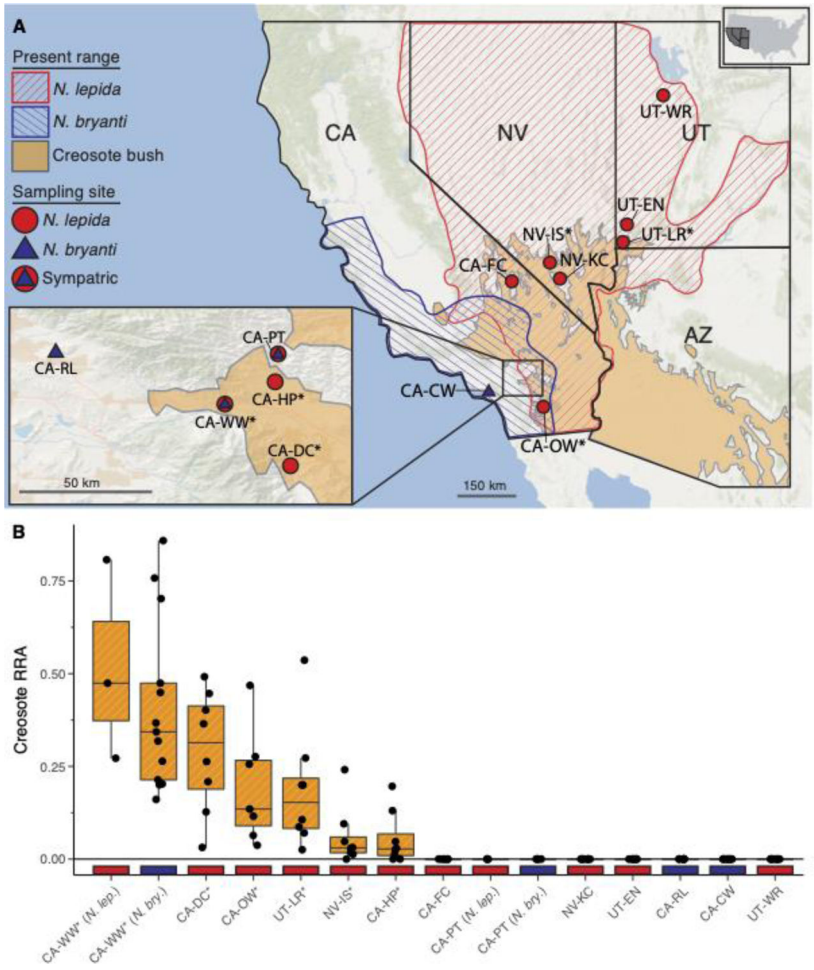

Figure 1.

(A) Map of ranges of *Neotoma lepida* (red hashing), *N. bryanti* (blue hashing) and creosote bush (brown) across the southwestern United States. Sites where woodrats were collected are shown with site codes and shaped by species (red circle = *N. lepida*; blue triangle = *N. bryanti*, sympatric sites = overlapping shapes). Woodrat ranges were obtained from the IUCN Red List (www.iucnredlist.org). Creosote bush range was obtained from Data Basin (www.databasin.org). (B) Plant metabarcoding results, with boxplots showing the proportion of reads identified as creosote bush (family Zygophyllaceae) in fecal samples. At sites where creosote bush occurred, creosote bush relative read abundance (RRA) varied between individuals and sites. Creosote bush was not detected in animals from sites without creosote bush. Sites colored based on *Neotoma* species (red = *N. lepida*, blue = *N. bryanti*).

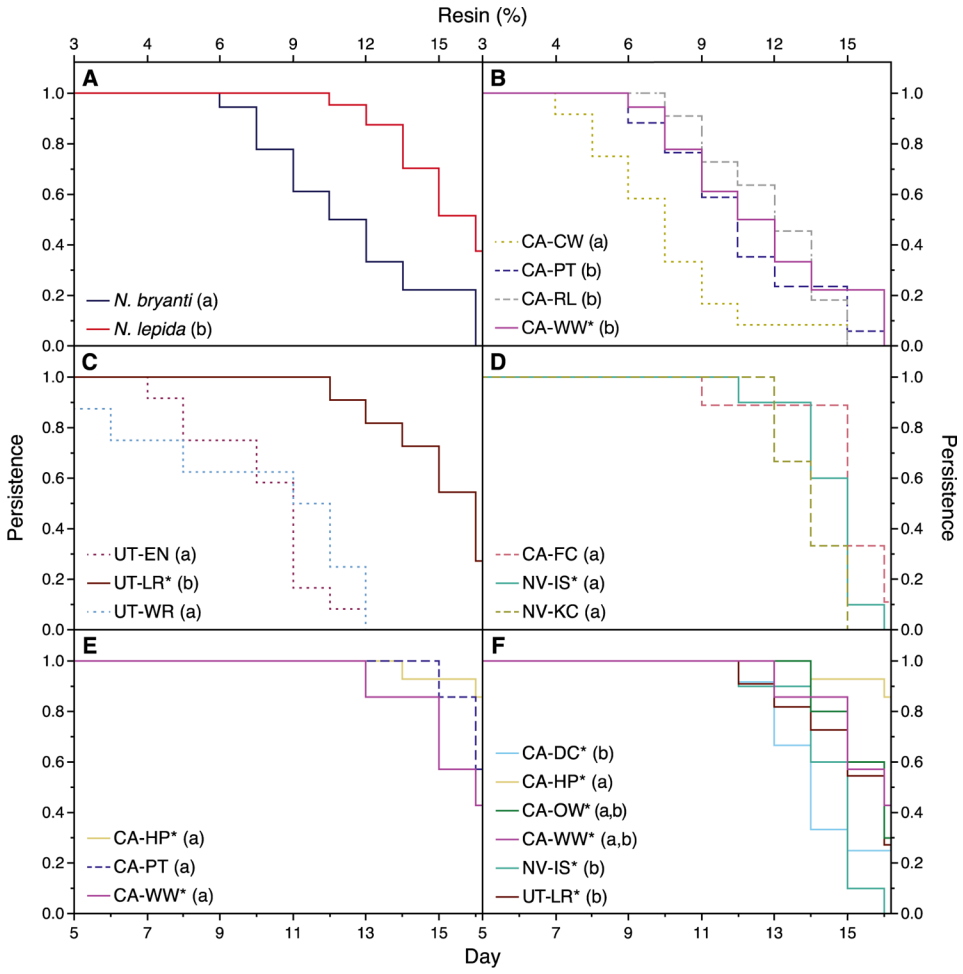

Figure 2.

Feeding trial persistence for *N. lepida* and *N. bryanti*. (A) *Neotoma lepida* and *N. bryanti* from creosote bush habitat differed in persistence. (B) Persistence for all four *N. bryanti* sites. Persistence for *N. lepida* fed creosote from (C) UT-LR*, (D) NV-IS* and (E) CA-HP*/CA-WW*. (F) Persistence for all *N. lepida* creosote-feeding sites. Dotted lines indicate sites over 25 km from the range of creosote bush, dashed lines represent sites within 25 km of documented creosote bush habitat, and solid lines indicate sites where creosote bush is present. Letters after site names denote significant differences in mean persistence.

Table 1.

Summary of field site localities and the captured animals used in this study.

Abbr.	Trapping location	Distance to creosote (km)	Elevation (m)	*N. lep.*	*N. bry.*	Feeding trial creosote
CA-CW	Casper's Wilderness, CA	71	300	–	12 (0)	CA-WW*
CA-DC*	Deep Canyon, CA	0	226	12 (3)	–	CA-DC*
CA-FC	Furnace Creek, CA	3	−37	9 (1)	–	NV-IS*
CA-HP*	Hoopa, CA	0	1,060	14 (12)	–	CA-HP*
CA-OW*	Ocotillo Wells, CA	0	137	10 (3)	–	CA-OW*
CA-PT	Pioneertown, CA	5	1,426	7 (4)	17 (0)	CA-HP*/CA-WW*
CA-RL	Redlands, CA	24	418	–	11 (0)	CA-WW*
CA-WW*	Whitewater, CA	0	441	7 (3)	18 (0)	CA-WW*
NV-IS*	Indian Springs, NV	0	1,175	10 (0)	–	NV-IS*
NV-KC	Kyle Canyon, NV	14	1,652	9 (0)	–	NV-IS*
UT-EN	Enterprise, UT	30	2,938	12 (0)	–	UT-LR*
UT-LR*	Lytle Ranch, UT	0	863	11 (3)	–	UT-LR*
UT-WR	White Rocks, UT	343	2,185	8 (0)	–	UT-LR*
			Total	109 (29)	58 (0)	

Elevation data was downloaded from the United States Geological Survey National Map website (https://apps.nationalmap.gov/). The counts of *Neotoma lepida* and *N. bryanti* individuals from each site that were involved in the feeding trials are given in *N. lep.* and *N. bry.*, respectively, with values in parentheses indicating the number of animals persisting to the end of the trial with less than 9.5% body mass loss. Feeding trial creosote identifies the source of creosote bush presented to animals from each abbreviated site (Abbr.). The presence of creosote bush at a particular locale is indicated by an asterisk (*) in the site name.